Research Article

Metabolic Engineering of Berberine in Plants Can Confer Resistance to Insects

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Abstract | Dimond Back Moth (*Plutella xylostella*) is highly destructive for cruciferous crops globally and resistant against different chemicals and biopesticides. DBM has shown resistance against 12 different strains of *Bacillus thuringiensis* (*Bt*) and 91 active ingredients of agricultural chemicals globally. Alternative ways should be explored to control DBM. Berberine is an insect toxic alkaloid and produced by plants of family *Berberidaceae*. *Berberine Bridge Enzyme* (*BBE*) gene involved in the synthesis of berberine was isolated from *Berberis lyceum* and over expressed in *Arabidopsis thaliana*. The integration of the *BBE* gene in transgenic lines was confirmed through PCR while the expression was confirmed by Reverse Transcriptase PCR. Whole plant bioassay confirmed 100% mortality in DBM within 72 hours. The present investigation concludes that the metabolic engineering of berberine in plants is an effective strategy to protect plants from herbivorous insects and this pathway should further be evaluated for possible application in agriculture.

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Introduction

DBM is chewing insect and belongs to order *Lepidoptera*. It is the universal insect and present all over the world where cruciferous hosts are present (Harcourt et al., 1962). DBM costs the global economy by estimated US$4 -5 billion annually and furthermore its impacts on local biodiversity and habitats in exotic ranges are unknown (Zalucki et al., 2012). In Pakistan, the cruciferous crops of entire regions of Punjab have been severely affected by this pest, furthermore this pest is frequently found in Hyderabad and Karachi regions of Sindh (Abro et al., 1994).

DBM is very destructive and most studied insect pest globally (Philips et al., 2014). Its control is very difficult, and it is the first insect that developed resistance to DDT (Ankersmit, 1953). Integrated pest management (IPM) approach has been developed which includes self-limiting gene technique, mating disruption and Sterile Insect Technique (SIT). Although these techniques are not harmful and pesticide free, but these methods are very costly and developing countries cannot afford these methods and frequent use of SIT is challenged by different hurdles such as sterilization through irradiation which affects the performance of insect and targeting only male members on large scale is another challenging task (Harvey-Samuel et al., 2015).

By transferring the insecticidal genes in a variety of cash crops have enhanced the crop yield and resolved the environmental issues. Recently number of insect resistant genes were isolated from various sources like animals, plants and microbes and incorporated in a

variety of crops that can induce resistance against major insect pests (Abbas et al., 2016). Developing of insect resistant cotton harboring *Bt* toxins is a well-known adopted technology globally (James, 2012; Qayyum et al., 2015). *Cry1Ac Cry2Ab, Cry 1F, Cry 2Ab* from *Bt* were effective against insect resistance but their frequent application lead to development of resistance in (Jackson et al., 2003; Gahan et al., 2005; Legwaila et al., 2014). Single gene in DBM has shown resistance against four different strains of *Bt* (Tabashnik, et al., 1997).

It is therefore advisable to explore novel genes to which insects have not been exposed so far in order to delay resistance in insect population and to minimize the crop losses. Berberine is an insect toxic alkaloid and produced by plants of family *Berberidaceae* (Gorval and Grishkovets, 1999). In response to wound latex berberine immediately spills out of leaves and binds to alpha 2 receptors. The complex of berberine and alpha 2 receptors adjust itself in between the synaptic cleft of nerve cells and cause the disruption of function of nervous system of insects (Schmeller et al., 1997). *BBE* is central enzyme catalyzes the oxidative cyclization of the N-methyl moiety of (S)-reticuline to the berberine bridge carbon C-8 of (S)-scoulerine that is further oxidized to berberine (Winkler et al., 2008, 2009). In the present investigation *BBE* gene was isolated from B. *lyceum* and over expressed in *A. thaliana*. The resultants transgenics were analyzed for toxicity against DBM. In present study it was investigated that can metabolic engineering of berbrine in plants be an effective strategy to protect plants from herbivorous insects?

Materials and Methods

Gene isolation and cloning

The nucleotide sequences of Berberine Bridge Enzyme genes were retrieved from NCBI and primers were designed from conserved region. The sequences of forward and reverse primers were 5'ATGATGTGCAGAAGCTTAACATTACGTT3' and 5'CTACAATTCCTTCAACATGTAAATTTCC3' respectively. Genomic DNA was extracted from the *Berberis lycium* using trizol reagent (Life Technologies Cat # 15596-018). The 50 ul Master Mix was prepared by mixing USB® FideliTaq mix ™ PCR Master Mix (2x), forward primer, reverse primer, ddH_2O and template DNA to the required concentration following the manufactures' instruction. PCR profile was comprised of initial denaturation for 5 minutes at 94°C followed by 35 cycles of 95°C for 1 minute, 50°C for 1 minute and 68°C for 2 minutes. Final extension was allowed for 7 minutes at 68°C. The amplified product was analyzed by gel documentation system. The amplified product was gel eluted using Gene JET gel extraction kit (K0692) and cloned in pTZ57R/T vector (Thermo Scientific Insta TA clone#K1214) according to the advised procedure and was sequenced from MACROGEN (Korea). The new isolated nucleotide sequence of the *BBE* gene was submitted to NCBI GenBank Accession No.MG601874.

Construction of expression vector

For construction of p35S-BBE-mYFP expression vector by gateway cloning, *BBE* gene was amplified from pTZ57R/T plasmid using primers containing attB sites at the 5'end. The sequence of forward primer was 5'GGGGACAAGTTTGTACAAAAAAGCAGGCT'ATGATGTGCAGAAGCTTAACATTACGTT3' and reverse primer was 5'GGGGACCACTTTGTACAAGAAAGCTGGGTCCTACAATTCCTTCAACATGTAAATTTCC3'. To generate the entry clone BP recombination reaction was carried out between attP sites of pDONAR™ 201 (Invitrogen) and attB sites of amplified PCR product. Further to develop the expression construct LR recombination reaction of attL and attR sites was performed between entry clone and destination binary vector pXCSG-mYFP. Designated p35S-BBE-mYFP construct was transformed into *Agrobacterium*strain GV3101 (pMP90RK) by electroporation.

Genetic transformation and screening of transformed plants of Arabidopsis thaliana

Floral dip method of transformation was used for genetic transformation of *Arabidopsis thaliana* as followed by Clough and Bent (1998). Infection was given after 30 days of sowing at maximum bolting stage. Seeds of T0 plants were harvested at maturity and T1 plants were screened through spraying BASTA (120 mg/L solution) twice a week. Transformation efficiency was observed 1.98%. Transformed plants were advanced to next four generations to get homozygous lines for BBE.

Molecular analysis of transformed plants

Transformed plants of every generation were confirmed through PCR using already mentioned primers and procedure. For transcript analysis, total RNA was

extracted from leaves of nine different transformed events and one wild-type isogenic line through Trizol reagent (Life Technologies Cat # 15596-018). First-strand of cDNA was synthesized from 10ng of RNA extracted from each transgenic and wild type isogenic line using oligo (dt) primers through AMV Reverse transcriptase. 2.5ul of resultant reaction product was used to amplify *BBE* gene in 50ul PCR reaction using 5'GTACGAGGTGGTGATGTT3' and 5'CAAGCACGGATACCGTAA3' primers of BBE gene. 18S rRNA forward primer 5'TGCATGGCCGTTCTTAGTTG3' and reverse primer 5'ATTGCCTCAAACTTCCGTCG3' were used as internal control.

Insect bioassays of transformed plants

Both the transformed (T3) and wild type isogenic lines were subjected to insect bioassays. Each line was exposed to 3 second instars larvae of *P. xylostella*. There were four replications and each replication was consisted of three plants. Weights of larvae before and after bioassays were compared. Mortality percentage of *P.xylostella* was also determined on transformed and wild type isogenic lines after 12, 24, 48 and 72 hrs of exposure. Statistix 8.1 was used to analyze the data. Least significant difference test (LSD) was carried to compare the means.

Results and Discussion

BBE gene isolation and cloning

DNA was extracted from leaves of *B. lyceum BBE* gene was amplified from DNA (Figure 1a) and cloned in pTZ57R/T vector. Plasmids were isolated from transformed white colonies (Figure 1b) and confirmed through PCR using already mentioned primers and conditions.

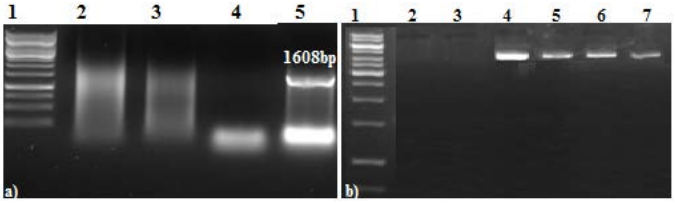

Figure 1: *Isolation and cloning of BBE gene. a) Agarose gel electrophoresis of amplified product from DNA of B. lyceum; b) Agarose gel electrophoresis of pTZ57 R/T harboring BBE gene.*

Developments of plant transformation construct

BP and LR reactions were carried out to develop a plant transformation construct. BP recombination reaction was carried out between attP sites of pDONAR™ 201(Invitrogen) and attB sites of amplified PCR product and entry clone was generated and confirmed through PCR (Figure 2a) LR recombination reaction of attL and attR sites was carried out between entry clone and destination binary vector pXCG-mYFP and plant transformation vector was developed (Figure 2b) and confirmed by PCR and sequencing. Physical map of plant transformation construct is given in (Figure 3).

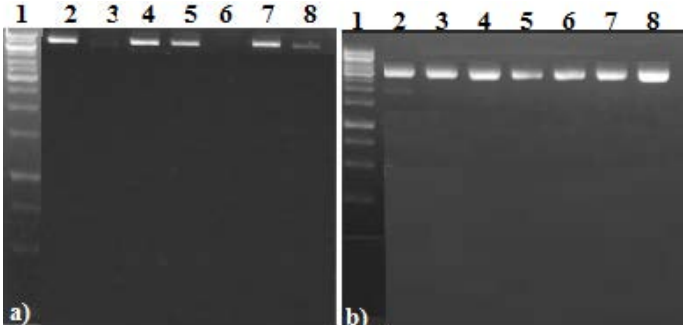

Figure 2: *Developments of plant transformation construct. a) Agarose gel electrophoresis of entry clone isolated from transformed colonies; b) Agarose gel electrophoresis of plant transformation vector isolated from transformed colonies.*

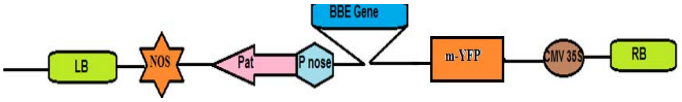

Figure 3: *Physical map of complete cassette. LB, Left boarder; RB, Right boarder; CaMV 35S, Cauliflower mosaic virus 35 S promoter; YFP, Yeast fluorescent protein; BBE, Cloned barbarian bridge enzyme gene; P nose, P nose promoter; Pat, Phosphinothricin acetyle transferase gene; NOS, NOS terminator.*

Transformation of Agrobacterium strain GV 3101(pMP90RK)

Designated p35S-BBE-mYFP construct was transformed into *Agrobacterium* strain GV3101 (pMP90RK) by electroporation and confirmed by PCR (Figure 4).

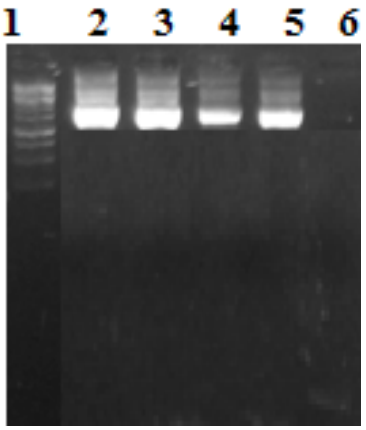

Figure 4: *Transformation of Agrobacterium strain GV 3101 (pMP90RK). Lane M, 1kb DNA ladder (Fermentas); lane 1 and 2, 3 and 4 are amplified products from transformed colonies of Agrobacterium Lane 6 is non transformed colony.*

Genetic transformation and molecular analysis of transformed Arabidopsis thaliana

The Photographic illustration of floral dip method is given in Figure 5. Muffins were prepared, and seeds were sown (Figure 5a). Infection was given after 30 days of sowing at the maximum bolting stage (Figure 5b and 5c). T1 plants were sprayed basta and survived plants were confirmed for transgene through PCR (Figure 6). Reverse transcriptase PCR showed that *BBE* gene was successfully transcribed in transformed lines while no such amplification was observed in non-transformed negative control plant. Expression of 18S rRNA gene in both transformed and non-transformed plant is shown in the lower panel of Figure 7. Results indicated that *BBE* gene is not only stably incorporated in the genome but also expressed to varying levels in different transgenic events (Figure 7).

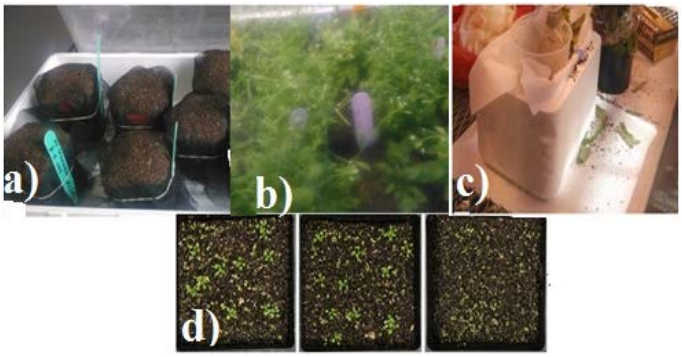

Figure 5: *Different steps of floral dip transformation. a, Muffin preparation and seeds sowing; b, Maximum bolting stage prior to infection with transformed Agrobacterium; c, Infection with transformed Agrobacterium culture; d, Screening of transformants with basta spray.*

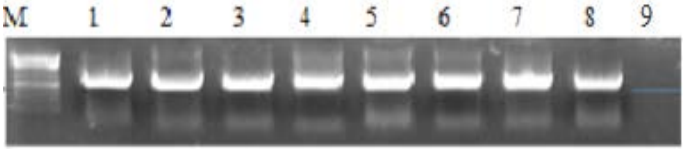

Figure 6: *Agarose gel electrophoresis of amplified products from transformed plants.*

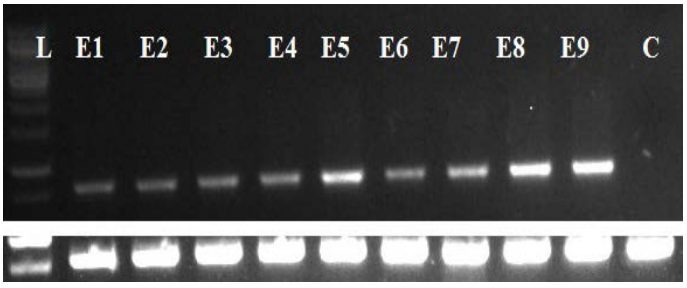

Figure 7: *Expression analysis of BBE gene. Lane E1 to E9, amplifications of BBE gene from the reverse transcription reactions product from different transformed events; lane C, no amplification of BBE gene from the reverse transcription reaction product of wild type isogenic line. Lower panel shows the expression of 18S rRNA gene.*

Insect bioassays of transformed plants

Statistical analysis revealed that the weight of 2nd instar larvae of DBM was significantly increased on wild type isogenic line after 72 hrs compared to larvae introduced on different transformed events (Table 1). The average weight of larvae increased from 0.56 mg to 1.125 mg on wild type isogenic line after 72 hrs of introduction while reduced weights were observed on different transformed lines (Table 1). Increased weight of larvae on wild type isogenic line after 72 hrs of introduction is shown in Figure 8b while reduced weights of larvae on different transformed events can also be observed in Figure 8d, 8f, and 8h.

Table 1: *Weight and mortality of 2nd instar larvae of Plutela xylostela.*

Lines	Larvae weight (mg)*		Mortality percentage time after introduction (h)				
	0	72	12	24	48	72	Means
NT	0.5613±0.003	1.125±0.014a	0	0	0	0	0.00
Line1	0.5633±0.003	0.243±0.001c	30	50	80	90	60DE
Line 2	0.5593±0.001	0.235±0.000b	60	70	80	96	76B
Line 3	0.5637±0.001	0.259±0.000b	15	10	50	80	43F
Line 4	0.5653±0.002	0.244±0.0017c	13	30	80	90	55E
Line 5	0.56±0.0005	0.244±0.002c	43	50	88	100	70BC
Line 6	0.5627±0.002	0.2577±0.002b	50	70	75	80	68C
Line 7	0.5613±0.001	0.236±0.000b	30	60	80	94	66CD
Line 8	0.562±0.0008	0.2427±0.001c	20	40	80	100	60DE
Line 9	0.559±0.0005	0.239±0.00cd	70	85	86	100	85A
Means	33.0D	48.0C	70.0B	83.8A			

**Mean of four replicates and each replicate was consisted of 3 insects; Means followed by same letters are not significantly different at p=0.05. $LSD_{0.05}$ for mortality percentage =6.62, $LSD_{0.05}$ for different lines =4.184.*

Different transformed events showed variable resistance to 2nd instar larvae of DBM and Line 5, 8 and 9 showed 100% mortality of DBM after72hrs and found more effective compared to other transformed lines (Table 1). Wild type isogenic line did not cause any death of 2nd instar larvae of DBM (Table 1). Dead larvae on different transformed events are encircled in Figure 8d, 8f and 8h.

Besides effective control on herbivorous insects, frequent application of synthetic pesticides has resulted in toxicity to non-target organisms, caused environmental pollution and harmful effects on human health (Damala, 2011; Gill and Garg, 2014). Environmentally friendly biopesticides based on *Bt* formulations were also extensively applied to control

herbivorous insects (Sauka and Benintende, 2008) but the natural ability of pests to evolve resistance challenged the benefits achieved by synthetic pesticides and biopesticides (Tabashnik, 1994). DBM is a highly destructive insect and has become resistant to all important classes of insecticides including *Bt* toxins.

Figure 8: *Insect bioassay of transformed and wild type isogenic line. a, wild type isogenic line exposed to 2nd instar larvae (photograph was taken just after introduction of larvae); b, wild type isogenic line (photograph was taken after 72 hours); c, e and g are transformed events 5, 8 and 9 respectively (photographs were taken just after introduction of insects); d, f and h are transformed events 5, 8 and 9 respectively (photographs were taken after 72 hours).*

Transgenic plants expressing single and double toxins are effectively used to control the damages caused by lepidopteron insects (Gahan et al., 2005). Each of Cry 2Ab and Cry1Ac toxin in transgenic Bollgard II cotton has unique receptor sites in the insects. This two-gene pyramid remained effective in delaying insect resistance because at least two independent mutations are required for insects to develop resistance against it (Jackson et al., 2003).

Due to the natural potential of insects to develop resistance, *Helicoverpa armigera* showed resistance to cotton expressing both Cry1Ac and Cry2Ab toxins (Ranjith et al., 2010). Field evolved resistance developed by herbivorous insects to different *Bt* toxins threatens the worth of *Bt* crops (Tabashnik and Carrière, 2009). Novel neurotoxic *Hvt* gene from *Hadrony cheversuta* was characterized for this purpose and transgenic expression confirmed its effectiveness against two lepidopteron insects (Khan et al., 2006). Translational fusion of *Hvt* from *Hadrony cheversuta* and *Cry1Ac* from *Bt* was developed and characterized for long lasting resistance in transgenic crops. Pyramiding distinctly related toxins from different sources has been an effective strategy to delay resistance and we further required to explore new toxins with unique mode of action to equip crop plants with more durable resistance against lepidopteron pests in future (Abbas et al., 2016).

Berbrine is the isoquinoline alkaloid (secondary metabolite) present in all members of *Berberidaceae* family (Gorval and Grishkovets, 1999). Berbrine is toxic to insects and restrains the growth of bactaria, fungi and viruses. It intercalates GC contents of DNA; affects the reverse transcriptase and DNA synthesis. Furthermore, these allelochemical activities are involved in chemical defense in response to microorganism, viruses and herbivores (Schmeller et al., 1997).

In current study the *BBE* gene (Central enzyme involved in the berberine synthetic pathway) was isolated from *B. lyceum* and over expressed in *At.* resultant transformed lines were assayed for toxicity against 2nd instar larvae of *P. xylostella.* Three transformed lines showed 100% mortality of larvae within 72hrs while other transformed lines also showed promising results with more than 80% mortality within 72 hrs. Reverse transcriptase PCR analysis showed more transcripts in transformed lines with 100 % mortality compared to others. Increased weights of larvae were observed on wild type isogenic lines compared to average weights of larvae noticed on transformed lines.

Our results suggest that over expression of novel *BBE* gene from *B. lyceum in At.* is a promising genetic resource to enhance the metabolic pathway of berberine biosyn thesis. No mortality of larvae in wild type isogenic lines and at least 80 % mortality in every transformed line is dedicated to enhanced production of berberine due to over expression of *BBE* gene in transformed lines. The study showed that transgenic expression of single gene in metabolic pathway has the potential to improve the production of useful plant secondary metabolites like (Berberine). It will delay resistance in lepedoptron insects especially in DBM as transgenic plants expressing toxins with unique mode of action from each other have more potential to delay resistance in insects (Abbas et al., 2013).

Berbrine Bridge Enzyme (BBE) is central enzyme catalyzes the oxidative cyclization of the N-methyl moiety of (S)-reticuline to the berberine bridge carbon C-8 of (S)-scoulerine that is further oxidized to berbrine (Winkler et al., 2008, 2009). Berbrine

accumulates in the lactifers in the form of latex (Schmelleret al., 1997; Yazaki et al., 2001; Shitan et al., 2003). Latex is a white sap which exudates from wound or point of damage caused by herbivorous insects (Lewinsohn, 1991). In response to wound latex berbrine immediately spills out of leaves and binds to alpha 2 receptors of insects. The complex of berbrine and alpha 2 receptors adjust itself in between the synaptic cleft of nerve cells and cause the disruption of function of nervous system of insects (Schmeller et al., 1997). Our study demonstrated that metabolic engineering of berbrine in plants is an effective strategy to protect plants from herbivorous insects and this pathway should further be explored for possible application in agriculture.

Acknowledgements

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Novelty Statement

The investigation corroborates the berberine to be an imperative tool having the crucial role toward the control of the herbivores and acquiring important positive fallout on crop improvement techniques through genetic manipulation and breeding approaches.

Author's Contribution

Anila Latif conceived, designed and conducted all the experiments during research. Dr. Ghulam Muhammed Ali Director NIGAB/ NARC supervised, facilitated and guided until the completion of research project. Dr Zaheer Abbas Senior Scientific Officer NARC, who has been the massive source of guidance, has always tried to remove hindrances that came while conducting the research. Dr Farhat Ullah Assistant Professor in AUP arranged the experimental data, proofread and finalized the manuscript.

Conflict of interest

There is no conflict of interest.

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